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(54) METHOD FOR PRODUCING A FINELY CRYSTALLINE OR AMORPHOUS SOLID STATE WHEN FREEZING SUBSTANCES SUCH AS BIOLOGICAL MATERIAL

(71) We, BALZERS PATENT- UND BETEILIGUNGS-AKTIENGESELLSCHAFT, a Liechtenstein body corporate, of FL-9496 Balzers, Principality of Liechtenstein, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a method for producing a finely crystalline or amorphous solid state when freezing substances containing water such as biological objects, and to an apparatus for carrying out this method.

In the production of preparations e.g. for electron microscopy it is of decisive importance that the structure of the objects investigated should not be varied or even destroyed. Substances which contain water, e.g. biological tissues, are usually firstly fixed by freezing, then the surface to be investigated is laid bare by means of a microtome, a metal is deposited thereon in vacuo from the vapor phase, if desired, for the purpose of increasing contrasts, and subsequently the investigation is carried out with an optical or electron microscope. Fixing by freezing has the known disadvantage, that the structure of the objects investigated is disturbed by the formation of ice crystals, and this the more the larger are the ice crystals forming. In order to attain a finely crystalline or if possible even amorphous solid state of the frozen water when carrying out the freezing, it has been proposed to effect the cooling as suddenly as possibly, for example by dipping the substances to be frozen or a container thereof into a liquefied gas such as liquid nitrogen as a coolant. In this manner one succeeds in attaining an amorphous or sufficiently finely crystalline condition (called vitrified condition) in an external marginal zone of the substance to be frozen, the thickness of which depends substantially on the kind of substance, its content in water, and the rate of cooling applied.

A method has been proposed according to which while cooling the substance to be frozen freezing is firstly suppressed by the application of a high pressure of about 2000 atm owing to lowering the freezing point, until a temperature of about −20°C is attained, whereafter by suddenly relieving the pressure a supercooled condition is established from which the substance freezes suddenly. Unfortunately it has been found that this known method of quick-freezing leads to a coarse crystalline ice structure. This formation of coarse crystals may perhaps be explained by the fact that the temperature of the substance to be frozen at the moment of freezing rises temporarily, owing to the latent of solidification liberated thereby and to the insufficient heat conduction, to a value at which the rate of crystal growth is strongly increased.

Experience has shown that most of the biological cell systems are inclined to a more or less high degree of possible supercooling before freezing. When the water surrounding the cells is incited to spontaneous crystal formation by a sudden pressure drop near its normal freezing point, there arises the possibility that the ice crystals formed extra-cellularly penetrate the cell walls and thus stimulate the intracellular water by heterogeneous seeding to form large crystals.

The present invention has the object of providing a method for producing a finely crystalline or amorphous solid condition when freezing substances containing water particularly biological objects, wherein the aqueous substance to be frozen is subjected to high pressure during part of the cooling period. The method according to the invention is characterised in that the pressure is maintained until the surface temperature of the substance to be frozen has dropped below −50°C.

The invention is based on the new discovery that for better attaining the desired object of vitrification as defined hereinabove

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of the substance the application of a high pressure during part of the cooling period is essential, and that the obvious course of pressure release for sudden initiation of the freezing at −20°C, putting up with the formation of coarse crystals, should be obviated.

The progress attained by the present invention may perhaps be explained in that by maintaining the high pressure up to a temperature T=−50°C, i.e. a temperature, at which the intracellular formation of crystallisation seeds of critical size has already reached a high degree, the subsequent reduction in pressure produces a repeated strong increase in the number of crystal seeds of critical size present in the cell bodies, with the result of a sub-microscopically fine ice structure. It then causes no trouble if the extra-cellular water frozen at a prior moment may have a coarser structure.

An embodiment of carrying out the method according to the invention, and a device suitable therefore will now be described in more detail by way of example with reference to the accompanying drawings, in which:—

Fig. 1 shows the essential portion of the device in detail, in longitudinal section, and

Fig. 2 shows the general arrangement, in longitudinal section, on a smaller scale.

In Fig. 1, 1 denotes a tubular thin-walled container closed at one end, consisting of a material of high thermal conductivity, e.g. copper, which receives the specimen 2 to be frozen. At its upper end 3 this container is an enlarged funnel-shape, and is clamped in between the cooling portion 4 and pressure portion 5 of the device. Through a central bore 6 of the portion 5 a high pressure of for example 1000 atm may be applied to the specimen by means of a transmission liquid —in the simplest case water—which pressure may be produced by means of devices known per se. The container extends into a chamber 7 provided with a supply conduit for the coolant formed by the passages 8, 9 and 10. When cooling, the coolant, when liquid, wets the outer surface of the container 1, rising in the chamber 7 until it overflows into the cavity 11 constructed as a receiving gutter of the portion 4, and eventually can escape through the ports 12.

Fig. 2 shows, how this device can be assembled with an auxiliary device for generating the pressure required. For this purpose, the portion 5 is supported by pillars 15, 16 fixed to a table 14, and is constructed as a hydraulic cylinder, the cylinder space 17 of which is in communication with the bore 6, and the hydraulic pressure piston 18 of which can be operated by the pneumatic piston 20 of a pneumatic cylinder through a stem 19. The pneumatic cylinder 21, the lower part of which only is shown, is provided in the usual manner with supply and discharge pipes, 22, 23 for the compressed air.

A frame 24 serves for supporting the pneumatic cylinder. 25 denotes a carrier platform guided on the pillars 15, 16 and which is slidable in the vertical direction by means of a hand wheel 26 and screw-threaded spindle 27, and serving as a support for the cooling portion 4. 28 denotes a pressure gauge serving for observation of the pressure applied to the specimen in the container 1.

In carrying out the method according to the present invention the specimen 2 to be frozen is introduced into the container 1 while the cooling portion 4 is lowered; then the latter is pressed against the pressure portion 5 by actuating the hand wheel 26, whereafter the specimen is subjected through the transmission liquid with a pressure between 100 atm and 1000 atm by means of the hydropneumatic device. While maintaining this pressure, the cooling medium, e.g. liquid nitrogen or a deep-cooled gas not yet condensed at the temperature desired, is introduced through the passages 8, 9, 10, whereby the specimen is cooled at a predetermined high rate. It is advisable to maintain this pressure when cooling until at least the marginal zone of the substance containing water is frozen, and in any case until the surface temperature of the substance to be frozen has dropped below −50°C, which may be checked e.g. by a thermo-electric element arranged on the specimen. Conveniently the relief of the pressure has to be delayed, until a practically complete equalisation of temperature has taken place between the coolant used and the object to be frozen.

Since the pressure, when lasting too long, may lead to the damaging of biological objects, it is advisable to use in this case during the cooling period a progressively rising i.e. variable pressure which at any moment of of the pre-cooling phase, i.e. above the predetermined solidification temperature, just suffices for preventing the freezing.

The magnitude of the pressure useful for carrying out the invention lies substantially between 100 atm and one thousand of atm. The pressure applied should be so high that a reduction of the freezing point of the substance containing water to be frozen of at least 5°C results.

In order to attain a sufficiently high rate of reduction in temperature, which is also of importance for carrying out the invention, it is advisable, as shown in the example described, to construct the container for the substance to be frozen as a partition wall of good thermal conductivity between the pressure chamber and cooling chamber, and to let the coolant flow into the cooling chamber at the highest possible rate when cooling. Since moreover, as likewise follows from the explanation above, the cooling of the specimen

and the application of the pressure have to be matched accurately in time, it is convenient to use an automatic switch gear for controlling both, which allows to adjust in an accurately controllable manner the relationship in time between the inflow of the coolant and the application of pressure. Without having to carry out at any time the difficult measuring of the surface temperature of the specimen, this time lag can be chosen on the basis of preliminary tests in such a manner that optimum vitrification of the specimen is attained. The control gear can be so constructed that the magnitude of the pressure varies in accordance with a preselected programme or in dependence of the temperature measured of the specimen.

The construction of such a control gear does not form the subject of the present invention.

The scope of application of the present invention is not limited to the aforesaid purpose of preserving the structure of biological objects of investigation, but includes also the preservation of high-grade biological substances, e.g. of blood plasma. The invention may likewise be applied for preserving the structure of inorganic substances with great advantage, e.g. in order to study the distribution of matter in solutions, suspensions, emulsions, gels or like mixed phases, or in order to fix such mixed phases by freezing for subsequent use. Also in cases, where a certain variation of structure may be put up with, the application of the invention may be of advantage, viz. in that the task may be fulfilled at considerably lower rates of cooling and accordingly more economically.

WHAT WE CLAIM IS:—

1. A method for producing a finely crystalline or amorphous solid state when freezing substances containing water such as biological objects, wherein the substance to be frozen is subjected to high pressure during part of the cooling period, said pressure being maintained until the surface temperature of the substance to be frozen has dropped below −50°C.

2. A method according to claim 1, wherein the pressure is maintained when cooling until at least an outer marginal zone of the substance containing water is frozen.

3. A method according to claim 1, wherein the pressure is maintained during the cooling period until a practically complete equalisation of temperature has taken place between the coolant used and the substance to be frozen.

4. A method according to claim 1, wherein a variable pressure is applied during the cooling period.

5. A method according to claim 1, wherein during the pre-cooling of the substance to be frozen to the predetermined freezing temperature the pressure is varied in such a manner that it just suffices at any moment of the pre-cooling period to prevent freezing.

6. A method according to claim 1, wherein a pressure of such magnitude is applied, that lowering of the freezing point of the substance containing water to be frozen by at least 5°C results.

7. An apparatus when used for carrying out the method according to claim 1 for producing a finely crystalline or amorphous solid state when freezing substances containing water such as biological objects, said apparatus comprising a container for the substance to be frozen, said container being provided with external cooling means and with means for applying high pressure inside said container until the surface temperature of the substance to be frozen has dropped to or below −50°C, which container is closed during the freezing operation.

8. An apparatus according to claim 7, wherein said means for applying high pressure comprise a hydraulic cylinder, the space of which is in communication with the said container, and the piston of which is operated by the piston of a pneumatic cylinder.

9. A method according to claim 1, substantially as hereindescribed.

10. An apparatus according to claim 7, substantially as herein described with reference to Fig. 1 of the accompanying drawings.

11. An apparatus according to claim 8, substantially as herein described with reference to Fig. 2 of the accompanying drawings.

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1230120 COMPLETE SPECIFICATION
2 SHEETS *This drawing is a reproduction of the Original on a reduced scale*
Sheet 1

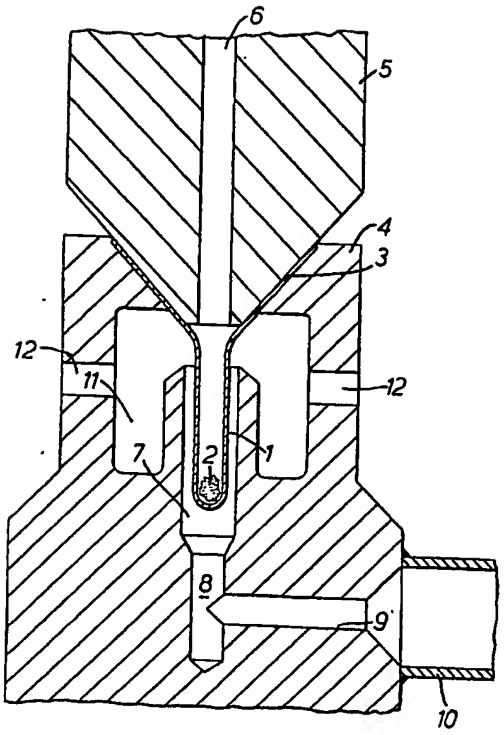

FIG. 1.

1230120 COMPLETE SPECIFICATION
2 SHEETS *This drawing is a reproduction of the Original on a reduced scale*
Sheet 2

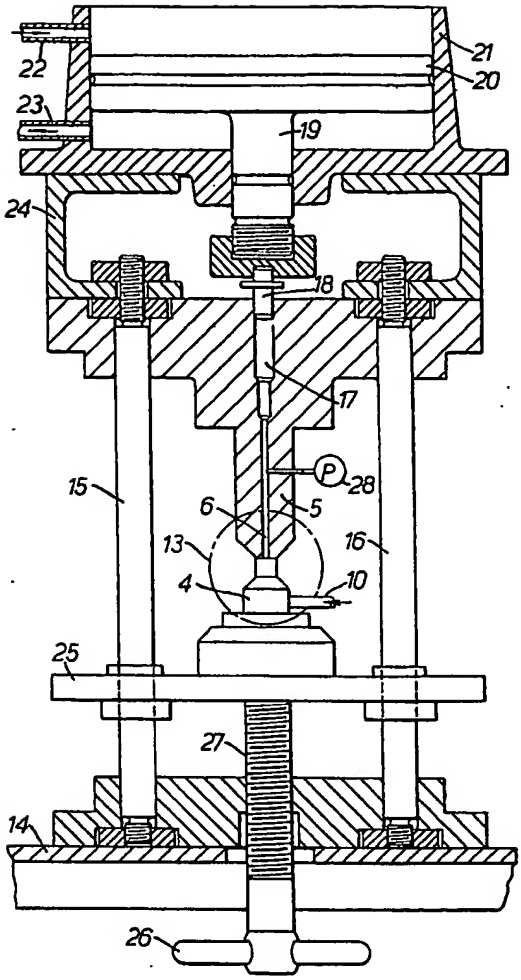

FIG. 2.